



## **Evaluating the Neuroprotective Effects of the Aqueous and Methanolic Extracts of *Vernonia amygdalina* on the Hippocampus of Adult Male Wistar Rats**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author USA designed the study and wrote the protocol. Author DKO wrote the first draft of the manuscript. Authors DKG, CSE and AEA managed the literature search. Authors FOO, KCO and DIO performed the statistical analysis. Author BNO, SFO, IJO and AJU managed the analyses of the study. All authors read and approved the final manuscript.*

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### **ABSTRACT**

The phytochemical yield of a plant material is related directly to the method as well as solvent of extraction. Lots of African herbs have proven over time to be highly medicinal both for the prevention, management and cure of various ailments. The aim of this research is to compare the

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neuroprotective potency of the aqueous and methanolic extracts of *Vernonia amygdalina* on the hippocampus of mercury chloride intoxicated Wistar rats. Thirty male Wistar rats weighing between 180 to 200 g were divided into 6 groups of 5 rats each. Group A was the negative control and had food and water only. Groups B was the positive control and groups C1, C2, D1 and D2 were the test groups. Rats in group B to D2 were exposed to 0.5 mg/kg/b.w of mercury chloride two times a week for 2 weeks. Groups C1 and C2 were treated with 200 mg/kg/bw of aqueous and methanolic extracts respectively while groups D1 and D2 received 400 mg/kg/bw of aqueous and methanolic extracts of *Vernonia amygdalina* respectively. Qualitative and quantitative phytochemical analysis shows that water extracted more flavonoids while methanol extracted more phenols from the plant. The results of antioxidant studies show that the methanolic extract conferred more protection against oxidative stress than the aqueous extract. Neurobehavioural and histological results show that aqueous extract conferred more protection on the cells and tissue structure of the hippocampus than the methanolic extract. We therefore conclude that both the aqueous and methanolic extracts of *Vernonia amygdalina* confer some form of neuroprotection on the hippocampus of Wistar rats but the aqueous extract gave better results.

**Keywords:** Methanol; aqueous; neuroprotective; hippocampus; *Vernonia amygdalina*; antioxidant.

## 1. INTRODUCTION

*Vernonia amygdalina* commonly called bitter leaf is of much interest due to its documented antioxidant and free radical scavenging properties. Reports show it is enriched with phenolic compounds which could be associated with its antioxidant and anti-inflammatory properties [1]. God'swill et al. [2] reported that the leaf of *Vernonia amygdalina* have a high content of carbohydrates, crude fat, crude protein, crude fibre, as well as a flavour which may enhance savoury and palatability of food. *Vernonia amygdalina* has been used in folkloric medicine for the treatment or management of several ailments. Phillipson et al. [3] and Ijeh & Ejike [4] reported the wound healing properties of *Vernonia amygdalina*. Iwalokun et al. [5] reported the anti-malaria effect of the plant. Akah and Okafor [6] and Osinubi [7] reported its anti-diabetic effect. Parts of these plants like the leaves, bark and roots are very rich in phytochemicals such as phenols, alkaloids, flavonoids, terpenes, glycosides with the inherent capacity to mitigate diseases and attenuate toxicities [8].

The method of extraction and type of solvent used are parts of the factors that determine the quality and quantity of the recovery yield and total phenolic content of the extracts [9]. Among these extracting solvents is water, ethanol, methanol, acetone, or their mixtures. Thus, the aim of this study is to evaluate the effect of different extracting solvents (water and methanol) on the recovery yield and total phenolic content of *V. amygdalina* leaf.

In recent years tremendous strides have been made to explore and put in scientific perspective the rich phytochemical and pharmaceutical properties of African herbs. African ancestors have used these herbs over the centuries to treat all manner of ailments. But due to poor packaging and unorthodox presentation, the younger generation began to tilt towards orthodox medicine in abandonment of the rich pharmaceutical heritage of our local herbs. Today, it is evident that Africans and the rest of the world have realized this rich heritage and therefore seek to harness it. Currently, several African countries including Nigeria and Ghana have a robust National programme for the development of herbs and formulation of herbal medicines [10].

With strong and emerging evidences of attempts to depopulate Africa through poisoned medications and vaccines, Africans as well as well-meaning global citizens must arise to the challenge of creating their own medicines from readily available herbal raw materials. It is difficult to explain the packaging of vaccines made for African children with mercury of all things, knowing the health dangers of exposure to mercury [11,12,13]. *Thimerosal is a mercury-based preservative that has been used for decades in multi-dose vials (vials containing more than one dose) of medicines and vaccines. In July 1999, the Public Health Service agencies, the American Academy of Paediatrics, and vaccine manufacturers agreed that thimerosal should be reduced or eliminated in vaccines as a precautionary measure. This was followed up and done accordingly. Over two decades after, this mercuric product is still used in making*

vaccines for African children, some of which are made free for easy access. According to the EPA the average child can tolerate 0.3 micrograms of mercury. However, a 2 month old infant receiving all 4 scheduled vaccinations (DtaP, Polio, Hib, Hepatitis B) will have a mercury level of 62.5 micrograms, 125 times higher than the EPA 'safe' level [13]. How also do we explain the vaccination of African children with DTaP (Diphtheria, tetanus, and pertussis vaccine for younger children) which was confirmed poisonous and banned from use in the United States over two decades ago [14]. The DTP vaccine was discontinued in the US and western nations in the 1990s following thousands of reports of death and brain damage. Girls vaccinated with the DTP vaccine died at 10 times the rate of unvaccinated kids. But they continued giving DTP and even made it a priority for African babies with the full backing of the world health organization. A study carried out in Guinea Bissau showed that 50% of children die before age five. In this West African nation, half the children were vaccinated with the DTP vaccine at three months and the other half at six months. The study found that girls vaccinated with the DTP vaccine died at 10 times the rate of unvaccinated kids [15]. While the vaccinated children were protected from Diphtheria, Tetanus and Pertussis, they were far more susceptible to other deadly diseases than unvaccinated peers. The researchers suggested that the DTP vaccine is killing more children than the diseases it targets [15]. On average about 15% of newborn children in Africa are expected to die before reaching their fifth birthday. The corresponding figures for many other parts of the developing world are in the range 3–8% and that for Europe is under 2% [16]. Data is poor as to the number of deaths in Africa based on the medicines administered. It is therefore expedient for Africans in particular and the world at large to rise up for our health against a minority who try to make merchandise at the expense of people's lives.

Oxidative stress has been linked with several ailments across the globe. Antioxidants on the other hand are compounds with the readiness to donate electron to electron-deficient free radicals to make them stable [17]. These free radicals in their quest to finding electrons attack cells and biomolecules of the body resulting and create abnormal functioning. The body in response generates natural antioxidants to mitigate the free radical load. However, it can be overwhelmed when the rate of production of free

radicals exceed the rate of mopping up by the body's defence. This leads to oxidative stress. There is therefore need to constantly augment the efforts of the body's natural antioxidants with external antioxidants. In doing this however, it is preferable to go for natural antioxidant sources like herbs. The use of synthetic antioxidants is not without harmful side effects [18]. It is therefore better to seek natural alternatives which are readily available and easily assessable especially to the African populace many of who otherwise may not afford the expensive and relatively dangerous ones.

Our ancestors who actively practiced herbal medicine ground or chopped the plant material into smaller pieces and soaked in water (aqueous) or ethanol (ethanolic) to serve as vehicles for ingestion of the extracts. People are told to shake vigorously, allowed to settle, and take shots of different sizes at different times of the day. This has been the practice over the years and Africans believe in its efficacy strongly. Our grandparents that lived most of their lives with these practices lived long, arguably longer than the present generation of Africans, hence the need to revisit this relatively declining practice.

## 2. MATERIALS

Materials used for this research includes but not limited to pipettes, syringes, sample bottles, haematology bottles, heparinized capillary tubes, cotton wool, methylated spirit, normal saline, light microscope, glass slides, 10% formal saline, test tubes, beakers (500 ml, 200 ml, 100 ml), distilled water, rat feed. The rats were housed in well-ventilated wooden wire gauzed cages with sawdust as bedding.

### 2.1 Collection and Authentication of Plant Material

Bitter leaf was obtained from Ekeoma market in Elele Rivers State and identified by Dr. EkekeChimezie, Department of Plant and Biotechnology, Faculty of Biological sciences, University of Port Harcourt, Nigeria with Herbarium number UPH/P/075.

### 2.2 Chemical

All chemicals used for this study were of analytical grade.

### 2.3 Experimental Animals

Thirty adult male Wistar rats of between 180-200 g were used for this study. The animals were purchased from the animal house of University of Port Harcourt. They were acclimatized for seven days and fed rat chow and tap water ad libitum.

### 2.4 Preparation of Plant Materials

The leaves of *Vernonia amygdalina* were plucked off their stalks, washed and air dried for four week under room temperature. The dry samples were then ground into powder with manual grinding machine.

### 2.5 Extraction Method

Four hundred grams (400 g) of the ground plant powder was soaked in bottles containing 1000 ml of distilled water for aqueous and 1000 ml of methanol for methanolic extracts for 24 hours. The mixtures were shaken intermittently for another two hours to allow complete extraction. The resulting mixtures were filtered using Whatman no. 1 filter paper to obtain a homogenous filtrate. The filtrate was then concentrated in vacuum at low temperature (37-40°C) using a rotary evaporator. The concentrates were allowed open in water bath (40°C) for complete dryness yielding about 30 g each of green gummy substance. The extracts were stored in a freezer at 2-8°C until use. Before use, extracts were reconstituted in distilled water at a concentration of 1 g/10 ml of distilled water prior to administration.

### 2.6 Preparation of Mercury Chloride Stock Solution

0.5 g of mercury chloride was dissolved in 125 ml of distilled water yielding 4 mg/ml (stock solution).

### 2.7 Experimental Design

Thirty male Wistar rats weighing between 180-200 g were used for this study. The rats were divided into 6 groups A, B, C1, C2, D1 and D2 of 5 rats each. Animals in group A received only food and water. All animals in the experimental groups B, C1, C2, D1 and D2 received intraperitoneally injections of mercury chloride (HgCl<sub>2</sub>) at 0.5 mg/kg/bw twice a week for two weeks according to the modified method reported on NTP (1993) [19] to induce toxicity.

Group B served as the positive control and received no treatment with *V. amygdalina*.

*Vernonia amygdalina* was administered orally throughout the research. Rats in the C-groups (C1 and C2) received 200 mg/kg per body weight of the aqueous and methanolic extracts respectively. Rats in the D-groups received 400 mg/kg per body weight of aqueous and methanolic extracts respectively.

After fourteen days of treatment, rats were subjected to Morris water maze test (day 15) and were subsequently sacrificed by cervical dislocation. The brains of the rats were harvested. Four of the brains were immersed into phosphate buffer solution at the rate of 1:4 for antioxidant studies. They were then homogenized and centrifuged at 10000rpm to separate the supernatant from the residue. The supernatant was used for the antioxidant assay. Antioxidant parameters tested include lipid peroxidation (MDA), super oxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT). The remaining brains from each group were fixed in 10% formal saline for histological studies using H&E method.

### 2.8 Neurobehavioural Studies

The Morris Water Maze (MWM) is a method of assessing spatial learning and memory (Morris).it was carried out according to the method of Stewart and Morris [20]. Animals were first trained in an initial trial (first trial) to escape drowning by climbing the escape platform. Then they are exposed to the real trial where the stage is submerged in plane water and in the last (used) trial. In this last trial the stage is removed and the water is made opaque with milk. The animals are tested on the time it will take them to locate the position of the escape platform. Only the result of the third trial was used for this work.

### 2.9 Anti-oxidant Assay

Brain samples from each group were taken for anti-oxidant tests to examine the activities of Superoxide dismutase (SOD), Catalase (CAT), reduced glutathione (GSH) and Malondialdehyde (MDA). The brain samples were collected and weighed. Phosphate buffer solution was prepared and poured into the specimen bottle containing each brain sample for homogenization using a homogenizing machine. The sample was centrifuged with cold centrifuge at 10,000 rpm (rounds per minute) and the supernatant was

collected. The rest of the assay was done using the supernatants.

A protein concentration was determined by the Biuret method described by Gornal et al. [21]. This is a pre-step in the determination of the antioxidant parameters. Lipid peroxidation was determined by the method of Varshney and Kale [22]. This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (MDA) – an end product of lipid peroxidation. The activity of SOD was determined by the method of Misra and Fridovich [23]. The level of reduced glutathione (GSH) was estimated using the method of Beutler et al. [24]. Catalase activity was determined according to the method of Sinha A., [25]. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H<sub>2</sub>O<sub>2</sub>, with the formation of perchromic acid as an unstable intermediate.

## 2.10 Histopathological Studies

After 48 hours of fixation, the brains were removed and the hippocampus excised from each one for histological assessment using H&E. Tissues were dehydrated in four changes of ascending grades of alcohol (70%, 80%, 90%, and absolute) for one hour each. Dehydrated tissues were cleared in two changes of xylene for 30 minutes each. Tissues were subsequently impregnated in four changes of molten paraffin wax at constant temperatures of 36-60°C in an oven of paraffin bath for one hour for each change. Metal blocks were filed with paraffin wax and tissues were quickly placed in it with forceps. When the paraffin cools, a paraffin block is formed and ready for sectioning. Thin sections were cut at 5µm using a rotary microtome after excess paraffin wax was trimmed off. Tissues were mounted on glass slides aided with albumin of egg. The section was put in the centre of the

slide so that the section starts floating. The section was immersed in water bath, keeping temperature between 50-55°C so that sections become straightened and wrinkles disappear. Water was drained off and the slide is put in an incubator so that the section is completely fixed on the slide and becomes dry. Tissues were stained with haematoxylin and eosin technique as described by Drury and Wallington [26]. Stained slides were focused under Leica research light microscope and photomicrographs taken from each group and labeled using Microsoft power point.

## 2.11 Statistical Analysis

Data were analyzed using Microsoft excel and one-way analysis of variance (ANOVA) and expressed as Mean ± SD of four rats per group. Differences between means were regarded significant at P < 0.05.

## 3. RESULTS

Table 1 shows that the aqueous extract of bitter leaf had higher quantity of flavonoids and alkaloids while methanol had higher saponins. Both had similar concentrations of phenols, tannins, steroids and glycosides.

From Table 2 above, it is seen that the methanolic extract had higher phenol, tannins and steroid counts compared to the aqueous extract.

The results of the phytochemical analysis above shows that considering phenol and flavonoid content which represent the antioxidant properties of a plant material, water extracted more flavonoids while methanol extracted more phenols. But from a general consideration, water gave more antioxidant phytochemicals (flavonoids plus phenol) than methanol.

**Table 1. Result of Qualitative Phytochemical Analysis of *Vernonia amygdalina***

Parameters	Aqueous	Methanolic
Saponins	++	+++
Flavonoids	+++	++
Alkaloids	+++	++
Phenols	+	+
Tannins	+	+
Steroids	+	+
Glycoside	+	+

NB: +++ Highly present, ++ Moderately present, + Low, - Not present

**Table 2. Result of Quantitative Phytochemical Analysis of *Vernonia amygdalina***

Parameters	Aqueous	Methanolic
Saponins	7.29	9.03
Flavonoids	6.58	4.38
Alkaloids	9.26	7.32
Phenols	0.78	0.95
Tannins	0.13	0.61
Steroids	0.15	0.28
Glycoside	0.41	0.37

**Table 3. Result of antioxidant studies**

GROUP	MDA	SOD	CAT	GSH
A	0.32±0.16	0.40±0.17	3.23±0.58	7.43±0.77
B	0.55±0.10*	0.15±0.07*	1.74±0.36*	3.72±0.54**
C1	0.46±0.11	0.20±0.09*	1.93±0.38*	3.80±0.30**
C2	0.43±0.06	0.31±0.08	2.59±0.70	4.30±0.62**
D1	0.45±0.15	0.28±0.06	2.04±0.24*	4.00±0.35**
D2	0.40±0.12	0.33±0.08	2.90±0.35	4.95±0.36**

Data is presented as Mean ± Standard deviation of 4 rats in each group. \*indicates statistical significance at  $P < 0.05$ , \*\*indicates high statistical significance at  $P < 0.05$

### 3.1 Results of Antioxidant Studies

The results of the antioxidant studies presented in Table 3 above indicated that malondialdehyde (MDA) levels were increased across all experimental groups compared to the control. However, only group B was statistically significant. Group B received 0.5 mg/kg/bw of mercury chloride intraperitoneally twice a week for the 2 weeks without treatment while groups C1 to D2 after mercury chloride intoxication received their corresponding extracts and doses of *Vernonia amygdalina*.

Superoxide dismutase (SOD) was reduced in all the experimental groups B, C1, C2, D1 and D2 compared to the control group A. The differences however were not statistically significant except in groups B and C1.

Catalase (CAT) also showed statistically significant reduction in the experimental groups B, C1 and D1. Although the others were also reduced compared to the control, their differences are not statistically significant.

Glutathione peroxidase (GSH) was highly significantly reduced in all experimental groups compared to the control group.

### 3.2 Results of Neurobehavioural Studies

Data was presented in minutes with reference to 60 seconds in one minute. Table 4 shows the result of Morris Water Maze test carried out on all 5 rats in the 6 groups used for this experiment.

INITIAL represent data collected after acclimatization before the commencement of mercury chloride administration while FINAL represents data collected at the end of the 2-week administration period.

There was on statistically significant differences between INITIAL AND FINAL for the control group A which received only food and water. However, for the group B which received mercury chloride only, there was statistically significant increase in FINAL compared to INITIAL.

**Table 4. Results of Morris Water Maze Test**

Group	Initial	Final
A	1.50±0.47	1.35±0.53
B	1.23±0.51	3.81±0.09**
C1	1.35±0.76	3.63±0.19**
C2	1.28±0.68	3.77±0.15**
D1	1.33±0.97	3.25±0.26*
D2	1.24±0.80	3.56±0.15**

Data is presented as Mean ± Standard deviation of 7 rats in each group. \*indicates statistical significance at  $P < 0.05$ , \*\*indicates high statistical significance at  $P < 0.05$

Also, there were statistically significant increases in the FINAL for the C-groups (C1 and C2) compared to their INITIAL although group C2 rats took longer time. For the D-group also, we see that although all the FINALS increased against their corresponding initials, D2 took longer time than D1. This implies that the aqueous extracts produced more positive results than the methanolic extracts in both doses administered.

significant differences in the means from the treatment groups C1 to D2 compared with that of the control group A at the INITIAL stage.

However, at the FINAL stage we saw highly significant increases in the means of results from the treatment groups compared to that of the control group A.

### 3.3 Results of Histological Studies

Table 5 presents a comparison across groups for INITIAL and FINAL results of Morris water maze test. Here again, there was no statistically

The results of the histological studies are presented in the plates below. Micrographs were placed side by side for ease of comparison.

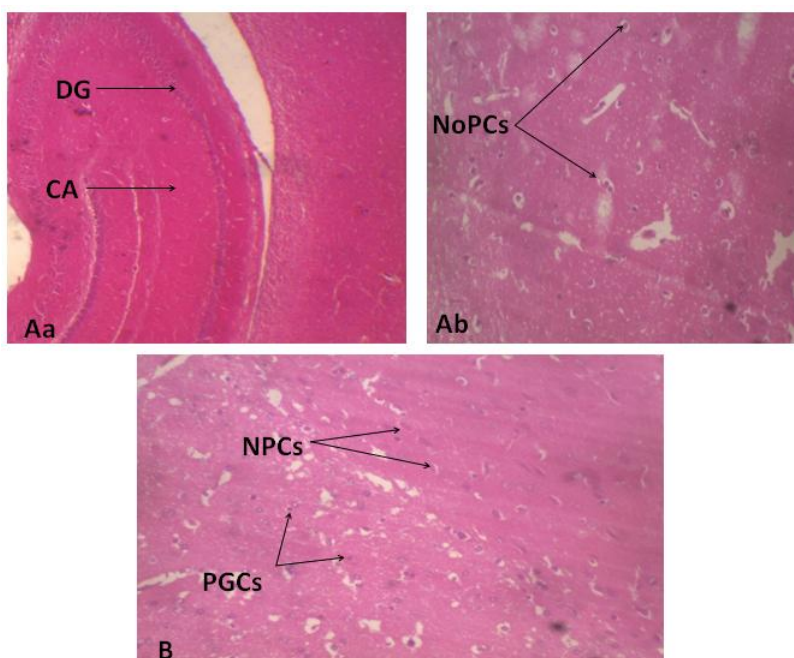
**Table 5. Comparing the results of the Morris Water Maze Test for INITIAL and FINAL readings across groups**

Group	A	B	C1	C2	D1	D2
INITIAL	1.50± 0.47	1.23± 0.51	1.35± 0.76	1.28± 0.68	1.33± 0.97	1.24± 0.80
FINAL	1.35± 0.53	3.81± 0.09**	3.63± 0.19**	3.77± 0.15	3.25± 0.26**	3.56± 0.15**

*Data is presented as Mean ± Standard Deviation of 5 rats in each group.*

*\*indicates statistical significance at P<0.05*

*\*\*indicates high statistical significance at P<0.05*



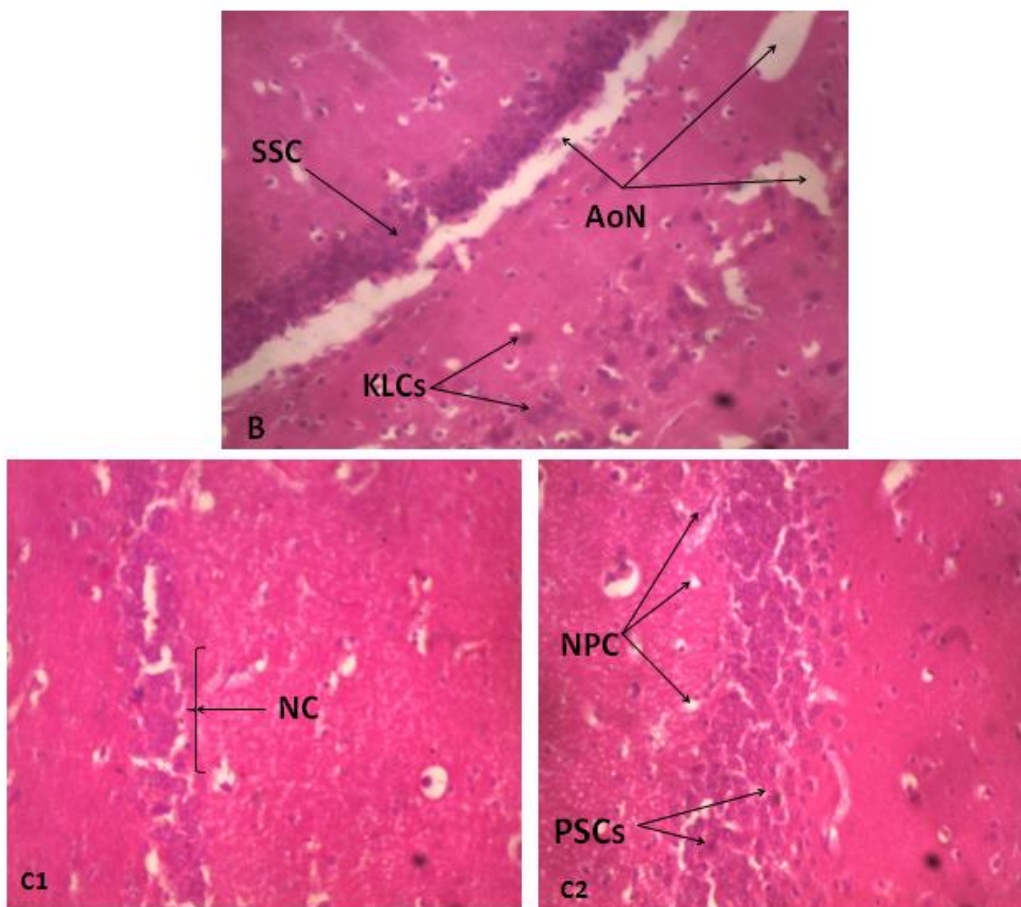
**Plate 1. Photomicrograph of rat hippocampus in group A (control) showing the dentate gyrus (DG) and the cornu ammonis (CA) at lower magnification (Aa). At higher magnification (Ab) we see normal pyramidal cells (NoPCs) with clear nuclear outline. We compare this with photomicrograph of rat hippocampus in group B showing numerous necrotic pyraminje (NPCs) and pyknotic granular cells (PGCs). These are signs of cell death**



#### 4. DISCUSSION

The presence of various antioxidant compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent [27]. Our result shows that the water (aqueous) extract of the leaf of *Vernonia amygdalina* had stronger neuroprotective and antioxidant effect compared to the methanolic extract. This may be attributed to the reported water solubility of some active phytochemicals in bitter leaf like flavonoids and phenols. Our phytochemical results however show that water and methanol extracts had nearly equal quantity of flavonoids while the methanol extracts more phenols than water. It is therefore noteworthy that contrary to expectations, the water extract had better antioxidant results in most of the

tested parameters than the methanolic extract, although in some, the differences were not statistically significant. Some other researchers had also reported that flavonoids and other phenolic compounds are potent water soluble antioxidants and free radical scavengers, which prevent oxidative cell damage [28,29,30]. Torel et al. [31] reported that flavonoids are good antioxidants, and luteolin (a flavonoid found in VA) has been reported to be a strong antioxidant. Furthermore, Nwanjo [32] reported that aqueous extract of *V. amygdalina* leaf significantly suppressed free radical induced oxidative damage. It was also reported that solvent polarity aid in increasing phenolic compounds' solubility [33]. God'swill [2] in his study reported that the aqueous extract of VA showed higher phenolic content than methanolic extract.



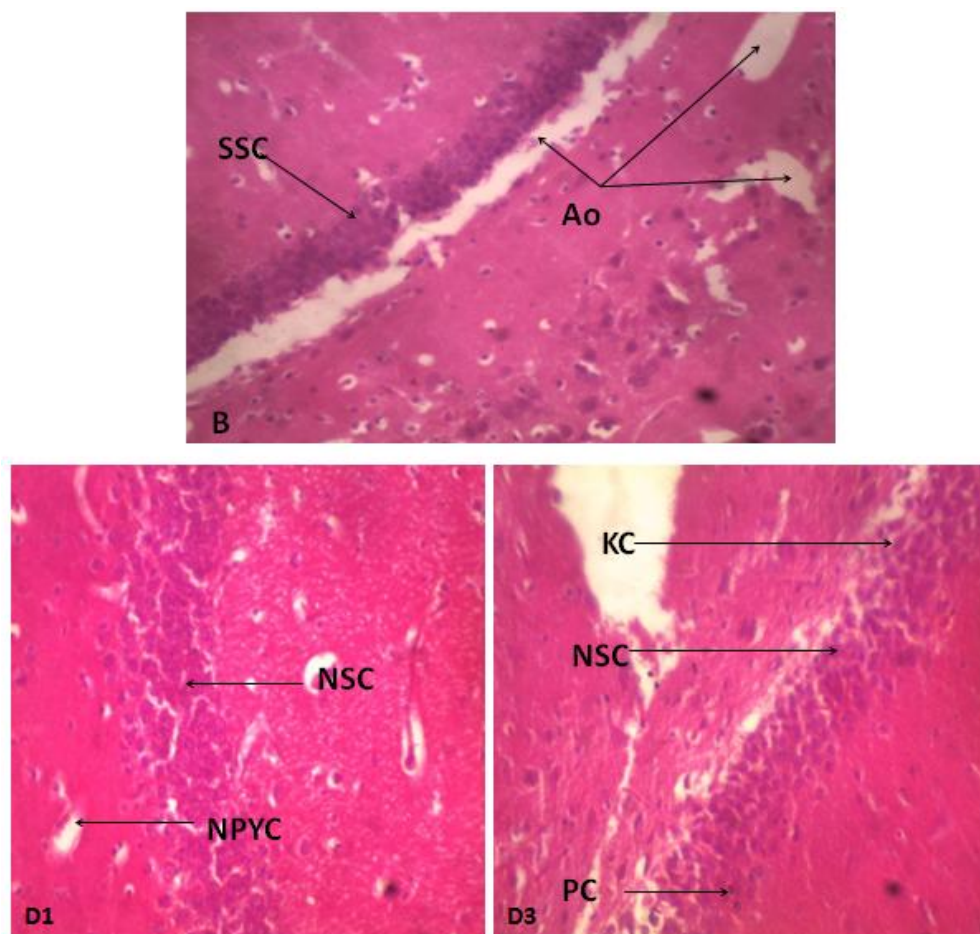
**Plate 2. Photomicrographs of rat hippocampus comparing the C-groups with the positive control group B. Group C1 shows relatively normal (NC) cytoarchitecture, although the cells appear fewer especially in the multiform layer X500. Group C2 shows relatively normal (NC) cell outline (X500)**



Differences in polarities of extracting solvents affects the solubility of chemical constituents in plants and thus extraction yield [34]. Norlia et al. [35] reported that the aqueous solvent was preferable for phenolic and flavonoid extraction from *Averrhoabilimbi* as opposed to methanol with lower polarity. Owoeye et al. [36] however reported that the methanolic extract of VA had higher neuroprotective ability on the cerebellum of gamma-irradiated Wistar rats. So we see that there may be no general rule as to which solvents should give a better phytochemical yield unless experimentally proven, because our work has shown that extraction yield with a particular solvent is not only plant dependent but also purpose dependent. So it is possible that water is the preferred solvent for bitter leaf extraction for

antioxidant purposes but methanol may be preferred for the same leaf for antimicrobial properties of the same plant.

Exposure to mercury continues to pose threats to the wellbeing of plants and animals, including humans. The result of our study shows that mercury chloride as low as 0.5 mg/kg/b.w of rat administered intraperitoneally twice a week produced toxicity on the rats. This was manifested through several physical signs of weakness including reduction in size, reduced food consumption and reduced general agility. We also saw evidence of oxidative stress (from the result of the antioxidant studies presented in Table 3 as well as the behavioural and histological changes.



**Plate 3. Photomicrographs of rat hippocampus comparing the D-groups to the positive control group B. Group D1 shows normal satellite cells (NSCs) and normal pyramidal cells (NPYCs). The hippocampal layers are also relatively normal although the satellite cells appear sequestered. X500. Group D2 shows a prominent area of necrosis (AoN). The satellite cells also appear relatively congestive. X500**

The result of this study shows that all the animals in the experimental group were under oxidative stress compared to the control. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the level the body's natural antioxidant defense mechanisms can cope with, causing damage to macromolecules such as DNA, proteins and lipids [37]. This is often characterized by high level of malondialdehyde (MDA) which is a marker of lipid peroxidation that occur following oxidative damage. The increase in the level of MDA is associated generation of free radicals, resulting in the peroxidation of membrane lipids.

For group B which did not receive any treatment following mercury chloride intoxication, there was significantly higher levels of MDA which is an indicator of lipid peroxidation. High MDA level is fallout of oxidative damage which could be linked to the generation of free radicals, resulting in the peroxidation of membrane lipids [38,39]. Reduced glutathione (GSH) is an endogenous antioxidant which plays a vital role in the detoxification of xenobiotics and scavenging of free radicals or reactive oxygen species (ROS) in cells [39]. A decline in cellular level has been considered to be indicative of oxidative stress. The decrease in this endogenous antioxidant in our experimental groups B to D2 supports the presence of oxidative stress in our experimental animals. Also reduced were levels of catalase (CAT) and super oxide dismutase (SOD). These trends of increasing MDA levels and corresponding decrease in SOD, CAT and GSH leave us with no doubt that mercury chloride administered twice a week intraperitoneally at 0.5mg/kg body weight of rat induced oxidative stress in the rats in the experimental group, especially when placed in the light of the results of the rats in the control group.

Following administration of bitter leaf extract, our result shows that the two extracts used for this research showed antioxidant properties in a dose dependent fashion. This is inferred following the results on table 3 which reveal that for all the tests carried out, the D groups (which received 400 mg/kg/b.w of corresponding doses of *Vernonia amygdalina*) performed better than the C groups (which received 200 mg/kg/b.w of corresponding doses of *Vernonia amygdalina*). This is supported by the work of Ayoola et al. [40] which reported that the efficacy of bitter leaf increased with increasing doses. Aguwa et al. [41] confirms that *Vernonia amygdalina* confers

protection on the brain against the adverse effects of mercury intoxication.

The level of lipid peroxidation (MDA) decreased in the experimental groups C1 to D2 following administration of the different extracts of *Vernonia amygdalina* compared to group B. It also showed that the values for MDA in the D groups were higher than those of the C groups. This represents a dose-dependent pattern of action. It means that the higher the dose consumed, the better the result obtained. This is supported by the work of God'swill et al. [2] which showed that both aqueous and methanolic extracts of VA prevented lipid peroxidation. Odukoya et al. [42] also reported the antiperoxidative effect of leaves of *Vernonia amygdalina*. Another study by Nwanjo [32] on diabetic rats showed that the aqueous extracts of VA decreased the levels of serum malondialdehyde.

The two extracts used for this study reduced the extent of lipid peroxidation. This was evident in the decreasing levels of malondialdehyde (MDA) in the treatment groups C1 to D2 when compared to group B which received no bitter leaf extract. Furthermore, the levels of the antioxidant enzymes which were significantly depleted in the experimental group B rose with *Vernonia amygdalina* administration in a dose-dependent manner. We are therefore convinced by these results in line with the finding of other researchers that *Vernonia amygdalina* is a potent antioxidant. It is reported that the antioxidant potential of a plant material usually appears to correlate with the phenolic content. Differences in the structure of phenolic compounds also determine their solubility in solvents of different polarity. Therefore type of extraction solvent as well as the isolation procedures may have a significant impact on the yield of extraction phenols from plants material [43,44]. Reports also show that plant phenolics are major group of compounds acting as primary antioxidants or free radical scavengers [45].

Mercury toxicity still remain a public health issue across different countries in the world especially the developing nations. In most of these nations, regulations are not properly followed up to ensure that industrial wastes are properly disposed of. So the land, water and air are polluted with dangerous amounts of mercury. This also goes on to contaminate drinking water, fishes and sea food, plants as well as poorly refined food. In fact, research shows that

vaccines are the highest source of mercury in children. Also, consumption of sea foods, especially fishes constitute some of the commonest means of exposure to mercury. They are also used as fungicides, antiseptics, preservatives, some electrodes and reagents [46] which are commonly used items. Anthropomorphic sources include the gold refining, smelters, cement production, sewage sludge incineration, municipal waste incinerators and fossil fuel combustion pollute the land [47].

Halas et al. [48] reported that mercury chloride administration cause zinc deficiency in the brain of rats. Bharnagar et al. [49] reported that zinc deficiency make glutamate produce inhibitory effect instead of excitatory, thereby distorting its function in learning and memory. Furthermore, Adamu [50] also reported reduction in levels of glutamate in the brain of rats exposed to mercury. Glutamate is the most abundant endogeneous amino acid in the mammalian central nervous system which functions as an excitatory neurotransmitter, but under abnormal conditions may behave as a neurotoxin, playing roles in the pathogenesis of neurodegenerative disorders that may lead to cognition impairment [49]. Halas et al. [48] also reported that decrease in zinc levels adversely affect the activities of hippocampal cells in memory formation, organization, storage and retrieval. These evidences lend support to our results which shows that mercury chloride not only elicited oxidative stress in the rats, but also led to cell deaths and cytoarchitectural distortion of hippocampal tissues. These toxicities were expressed as the rats performed poorly in the neurobehavioural tests where they actually performed relatively better before being exposed to mercury chloride. Reports from the Agency for toxic substances and Disease Registry [51] shows that exposure to mercury causes symptoms such as tremors, nervousness, memory loss and neuromuscular changes including muscle atrophy and weakness. Rats under such health conditions will not perform well in the neurobehavioural tests we conducted, hence supporting the outcome of our research.

The toxicity of mercury chloride is long established through various scientific reports. Mercury chloride affects different organs and systems of the body, including the nervous system. Barregard [52] reported on the toxic effect of mercury on blood count; Rao et al., [53] reported on the liver; Hallee [54] and Goyer [55] on the lungs; Rice et al. [56] and Clarkson [57]

reported on the gastrointestinal tract, Pambor et al. [58] and Goh [59] reported on the skin and integumentary system; Thurtson et al. [60] and Altman [61] reported on the heart and cardiovascular system; Booth [62] reported skin rashes, dim vision and clamminess; Rao & Sharma [63] and Baltimore [64] reported on menstrual disturbances and pregnancy complications following exposure to mercury through different routes.

The result of the Morris water maze test used to test for learning and memory showed that after exposure to mercury, the rats in the experimental groups B took longer time to discover the escape platform when compared to the rats in the control group. This could be as a result of memory loss or difficulty establishing new memories. This outcome followed mercury chloride administration. Impairments in spatial learning and memory are associated with damage of hippocampal cells. Here again, this is expected of rats in group B as they received mercury chloride without treatment. The thrust of this research is to see the impact of administering bitter leaf extracts to the rats after exposure to mercury. All the rats in the treatment groups C1-D2 also experienced statistically significant increases in the length of time it took them to locate the escape platform. We however observed that between the experimental groups C1 & C2, the rats in group C1 took relatively shorter time to locate the platform compared to rats in group C2 although the difference was not statistically significant. This is also the same for group D1 compared to D2. We recall that C1 and D1 received the aqueous extract of bitter leaf while C2 and D2 received methanolic extracts of *Vernonia amygdalina*. The results therefore suggest that the aqueous extract of *Vernonia amygdalina* had more protective outcomes on the learning and memory and by extension on the hippocampus of the experimental rats compared to the methanolic extracts. Aguwa et al. [41] reported that exposure to mercury negatively impacted on rats performance on the hanging wire test.

The increase in time taken by the experimental rats to find the hidden platform in the Morris water maze test, for memory and learning may be due to decrease in concentration of zinc induced by exposure to mercury chloride. Studies by Mutter et al. [65] showed that depletion in zinc levels may lead to decrease in the modulatory role of zinc on the neurotransmitter glutamate that plays a vital role

in memory and learning. Zinc deficiency was associated to memory loss among Alzheimer's disease patients and also among workers exposed to mercury vapour for 20 years [65]. Olson [66] had reported that short term occupational exposure to high levels of mercury induced slight cognitive deficits. Our result however contradicts the report by Mutter et al. [65] who researched on cognitive test using Y-maze for memory and showed that exposure to mercury has no effect on memory.

We also carried out histopathological studies on the hippocampus. The histology results as shown in plates 1 to 3 reveal that mercury chloride administration produced deleterious effects on the cells and cytoarchitecture of rat hippocampus. The general degenerative changes in the hippocampus were observed as degenerated areas, necrotic cells and distortion of cellular layers of the hippocampus. The pyramidal cells of the hippocampus were adversely affected, showing several signs and different stages of necrosis, including pyknosis, karyolysis and karyorrhexis.

The pyramidal cells in the hippocampus showed signs of cell degeneration and reduction in number, to loss of neuronal cell fibre due to the reduced number of cells when compared to the control group. This can be attributed to exposure to mercury chloride as these manifestations were not present in the control group. This implies that the activity of the hippocampus in memory formation and learning may be impaired and the role of the hippocampus that involves storage and retrieval of information may also be adversely affected. The findings in this study agrees with the work of Wolf et al., [67] who reported that rats exposed to high concentration of mercury vapour, showed neurodegenerative changes in the hippocampus which was responsible for memory deficit in such animals. Also, Gagelli et al. [68] had shown that cell sizes and numbers were decreased in mice treated orally with inorganic mercury at high doses for a week.

Onwureme and Oladimeji [69] reported that mercury exposure causes histopathological changes in the hippocampus of *Oreochromis niloticus* exposed to waste products from Nigeria National Petroleum Company (NNPC) Kaduna refinery of which mercury exposure is a major part. It is therefore not surprising that the animals in the experimental groups performed poorly in the Morris water maze test carried out after the

period of treatment to test for hippocampal function compared to the results obtained before exposure to mercury chloride.

## 5. CONCLUSION

Both aqueous and methanolic extracts of *Vernonia amygdalina* confers protection on the hippocampus against mercury chloride induced oxidative stress. However, aqueous extract conferred more protection than its methanolic counterpart.

## DISCLAIMER

The plant material used for this research grows freely in the region where the study was carried out. Also the research was solely funded by the authors.

## ETHICAL APPROVAL

Due ethical approval was gotten from the research ethics committee of the Faculty of basic Medical Sciences, Madonna University Nigeria. Clearance Paper is scanned and attached.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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